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Articles

Tolerance of *Cordia americana* plants exposed to excess copper

Tolerância de plantas de *Cordia americana* expostas ao excesso de cobre

Luana da Rosa Lovato[I], Marcos Vinícius Miranda Aguilar[I], Flaiane Catarine Rosa da Rosa[I], Thomas Wink Peixoto[I], Gerâne Silva Wertonge[I], Daniel Vinicios Valsoler[I], Tais Dorneles de Azevedo[I], Lucas Caye da Silva[I], Luciane Almeri Tabaldi[I]

[I]Universidade Federal de Santa Maria, Santa Maria, RS, Brazil

ABSTRACT

Copper (Cu) is an essential micronutrient for plants; however, it contaminates air, water, and soil in high concentrations. Phytoremediation is an alternative to using plants that are tolerant to excess metals in the soil. The study aimed to assess the tolerance of *Cordia americana* species to excess Cu using morphological, physiological, and biochemical variables. The plants were grown in a greenhouse under five Cu concentrations (0 (complete nutrient solution), 15, 30, 45, and 60 μM) in the nutrient solution. Twenty trays (16 liters each) were used, with five plants per tray. The morphological variables of the shoot and root system, photosynthetic variables, chlorophyll a fluorescence, photosynthetic pigments, antioxidant enzyme activity, hydrogen peroxide content, and Cu content accumulated in the tissues were evaluated. Root morphology, photosynthetic variables, chlorophyll a fluorescence, and photosynthetic pigments were not negatively affected by adding Cu to the cultivation system. Biochemical analyses demonstrated that the species used defense techniques against excess Cu, accumulating the metal in its root system and preserving the shoot for photosynthesis. The growth in shoots and roots and biomass production were not affected by increasing Cu concentrations. Therefore, the *Cordia americana* species has the potential to be used in areas contaminated with Cu.

Keywords: Guajuvira; Metal toxicity; Morphophysiological variables; Oxidative stress; Phytoremediation

RESUMO

O cobre (Cu) é um micronutriente essencial para as plantas, porém, em altas concentrações, contamina o ar, a água e o solo. A fitorremediação entra como alternativa à utilização de plantas que sejam tolerantes ao excesso de metais no solo. O objetivo do estudo foi testar a tolerância da espécie *Cordia americana* ao excesso de Cu, utilizando variáveis morfológicas, fisiológicas e bioquímicas. A espécie foi cultivada em casa de vegetação, em cinco concentrações de Cu (0 (solução nutritiva completa), 15, 30, 45 e 60 µM) na solução nutritiva. Foram utilizadas 20 bandejas de 16 litros, com 5 plantas por bandeja. Foram avaliadas as variáveis morfológicas da parte aérea e do sistema radicular, variáveis fotossintéticas, fluorescência da clorofila *a*, pigmentos fotossintéticos, atividade de enzimas antioxidantes, conteúdo de peróxido de hidrogênio e teores de Cu acumulados nos tecidos. A morfologia das raízes, as variáveis fotossintéticas, fluorescência da clorofila *a* e pigmentos fotossintéticos não foram afetados negativamente mediante acréscimo do Cu no sistema de crescimento. As análises bioquímicas demonstraram que a espécie usou técnicas de defesa contra o excesso de Cu, acumulando o metal em seu sistema radicular e preservando a parte aérea para a fotossíntese. O incremento em parte aérea e raízes não foi afetado com o aumento das concentrações de Cu, bem como a produção em biomassa. Sendo assim, a espécie *Cordia americana* possui potencial para ser utilizada em áreas contaminadas com Cu.

Palavras-chave: Guajuvira; Toxidade de metais; Variáveis morfofisiológicas; Estresse oxidativo; Fitorremediação

1 INTRODUCTION

After the Industrial Revolution and the increase in population, environmental pollution began to intensify, generating questions about future environmental problems and raising long-term concerns. Allied to this, atmospheric contamination in soils and water resources by heavy metals were some of the processes that became recurrent due to anthropization (Aguilar *et al.*, 2023).

Soils contaminated by heavy metals have toxic effects on plants, animals, and humans. Among these heavy metals that can contaminate soils is copper (Cu), which is an important micronutrient for plant growth, as it is related to processes such as photosynthesis, carbohydrate and nitrogen metabolism, and respiration (Lunkes *et al.,* 2022), but it must be present in the soil in low concentrations. However, some soils may present Cu toxicity, causing complications for seedling growth, as it directly affects root growth, potentially inhibiting the adsorption of nutrients, and causing changes in physiological and biochemical processes (Ambrosini *et al*., 2016). Excess Cu can also cause damage to the sulfhydryl groups of proteins and induce the peroxidation of lipids in cell membranes (Silva *et al*., 2011), in addition to generating reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), causing imbalances inside the plant and damage to DNA and biomolecules (Silva *et al*., 2011).

Rio Grande do Sul (Brazil) already has many reports of soils contaminated by Cu (Silva *et al*., 2011). The State is historically known for being the largest national producer of grapes, with approximately 51% of production centralized in the Serra Gaúcha region (Ayres *et al*., 2022). However, high temperature, humidity, and rainfall levels result in abundant fungal diseases in grapevine plants, which are prevented by using fungicides (Ambrosini *et al*., 2016). The fungicides used to control diseases in grapevines are based on Cu, so the soils in these areas are extremely contaminated by excess Cu, affecting production (Ambrosini *et al*., 2016).

Therefore, the use of plant species to extract, contain, transfer, stabilize, or degrade organic compounds and toxic metals, which is called phytoremediation (Leite *et al*., 2019), can be an interesting and important strategy to remedy these contaminated soils since the high accumulation of biomass and the dense root system of some tree species can immobilize metals in their tissues (Covre *et al*., 2020).

Species with desirable characteristics for phytoremediation must be chosen, such as fast growth, high biomass production, tolerance and/or adaptability to polluted soils, and a deep root system so that the roots absorb the metal present in deep layers of the soil (Silva *et al.,* 2019).

In this context, *Cordia americana* (L.) Gottschling & J.S.Mill. is a species that presents desirable characteristics for phytoremediation, as it presents extensive root growth and biomass production. Furthermore, this species is used to recover degraded areas, as it withstands periodic flooding and is widely used to contain river embankments (Carvalho, 2003).

C. americana is a semi-deciduous species native to Brazil, belonging to the Boraginaceae family. In some states, such as Rio Grande do Sul and Paraná, it is popularly known as Guajuvira (Carvalho, 2003). This species has simple, alternate leaves and a slightly longitudinally fissured trunk. *C. americana* can be used for various purposes, such as landscaping streets, squares, and parks, as it has beige or white, fragrant flowers. Furthermore, the species has a high potential for sawmilling (sawn and round wood) and for energy production, as it has firewood that is easy to burn (Carvalho, 2004).

Given the above, the present study aimed to evaluate how the *Cordia americana* reacts to different Cu concentrations of Cu in growth medium to determine its potential tolerance and/or sensitivity to Cu. By analyzing morphological, physiological, and biochemical variables, it is possible to understand whether the species will be able to adapt to environments contaminated by Cu and reduce the bioavailability of this metal.

2 MATERIALS AND METHODS

2.1 Study location and plant material

The present study was conducted in the greenhouse of the Department of Biology at the Federal University of Santa Maria in Santa Maria – RS (29°42’56.35” S and 53°43’12.64” W), with a controlled temperature of 25ºC and relative air humidity of 60%.

C. americana seeds were collected from parent trees located in the central region of Rio Grande do Sul and produced by the UFSM Forest Nursery (Campus Santa Maria). Sowing was conducted in single-cell trays with commercial substrate Carolina Soil® composed of peat moss (*Sphagnum* spp.), vermiculite, and 30% carbonized rice husk. Then, the germinated seedlings were transferred to polypropylene plastic tubes with a volume of 180 cm^3 using the substrate mentioned above. At 90 days after sowing, and with approximately 25 cm in height, the seedlings were sent to the greenhouse of the Department of Biology at the Federal University of Santa Maria in Santa Maria (RS), Brazil.

2.2 Experimental conduction

The experiment was conducted in a completely randomized design. The species was conducted under five copper concentrations, with four trays per concentration and five plants per tray. Thus, 20 16-liter trays were used, totaling 100 plants evaluated. The treatments had the following Cu concentrations: 0 (complete nutrient solution), 15, 30, 45, and 60 μM. The concentrations above were added to the complete nutrient solution for Cu treatments. The complete nutrient solution used in the study was proposed by Hoagland & Arnon (1950), with the following composition in mg L^{-1}: NO_3^- = 196; NH_4 = 14; P = 31; K = 234; Ca = 160; Mg = 48.6; S = 70; Fe-EDTA = 5; Cu = 0.02; Zn = 0.15; Mn = 0.5; B = 0.5; Mo = 0.01.

The seedlings were removed from the tubes, and their roots were washed in distilled water with extreme care not to damage them. Then, Styrofoam plates with openings were added to the trays to accommodate the five plants. The Styrofoam plates prevent further evaporation of the solution and simultaneously guarantee the fixation of the seedlings in the trays. Furthermore, the roots were aerated with PVC microtubes connected to a compressor to supply oxygen to the root system. The plants were maintained in acclimatization in the nutrient solution until the production of new roots was observed, which totaled 50 days of acclimatization in a hydroponic system. The plants were exposed to copper for 32 days, totaling 82 days of cultivation in a hydroponic system.

The nutrient solution was changed once a week during the experiment. For each treatment, hydrochloric acid (HCl) solutions (diluted in distilled water) were used to make the pH more acidic, and sodium hypochlorite (NaOH) solutions (diluted in distilled water) were used to make it more basic, that is, increasing or decreasing pH respectively until it stabilized at 5.7.

Plants were collected after 32 days of Cu exposure in a hydroponic system when they showed visual symptoms of Cu toxicity, especially at the highest Cu concentration (Figures 1 and 2, supplementary material).

2.3 Morphological variables

Two plants were measured per replication to analyze growth variables, totaling eight plants per treatment. Thus, the size of the shoot and main root length of *C. americana* plants were measured with a millimeter ruler before Cu was added and at the end of the experiment period, that is, when the plants were collected. In this way, it was possible to obtain data on the increase in main root length (IR) (Equation (1)) and the increase in shoot height (SHI) (Equation (2)), subtracting the final size (with Cu) of the seedling from the initial size (without Cu), in cm, according to the following formula.

$$IR = (\text{Final length of main root} - \text{Initial length of main root}) \quad (1)$$

$$SHI = (\text{Final shoot height} - \text{Initial shoot height}) \quad (2)$$

The seedlings intended for growth analysis were separated into shoots and roots at the end of the experiment. Subsequently, the roots were carefully washed with distilled water, wrapped in paper towels (soaked with distilled water), and placed in the freezer. The roots were scanned with the WinRhizo Pro 2013 software, coupled with the EPSON Expression 11000 scanner equipped with additional light (TPU), with a resolution of 600 DPI. The software generated the variables total root length (cm), root diameter (mm), root volume (cm^3 $plant^{-1}$), and root surface area (cm^2 $plant^{-1}$).

After scanning the roots, the root and leaf samples were then dried in a forced air circulation oven (65 ± 1°C) until a constant weight was reached, obtaining results of shoot dry weight (SDW) (g $plant^{-1}$) and root dry weight (RDW) (g $plant^{-1}$).

2.4 Chlorophyll a fluorescence

Chlorophyll a fluorescence was analyzed using a portable modulated light fluorometer (Junior-Pam Chlorophyll Fluorometer Walz Mess-und-Regeltechnik, Germany). For this, on a sunny day, from 8 am to 9:30 am, measurements were taken on fully expanded leaves of the plant. The leaves were exposed to the dark for 30 minutes so that their pre-adaptation could occur and the initial fluorescence (Fo) could be collected with Junior-PAM Chlorophyll Fluorometer. Then, in a period of 0.6 seconds, the leaves were exposed to a pulse of saturating light (10,000 μmol m^{-2} s^{-1}) to determine the maximum fluorescence (Fm). The electron transport rate (ETR) was determined from the fluorescence induction curve (1,500 mmol m^{-2} s^{-1}). The ratio between the variable fluorescence (Fv = Fm-Fo) and the maximum fluorescence was calculated to estimate the maximum quantum yield of photosystem II (Fv/Fm).

2.5 Analysis of Cu in plant tissues

After being dried in an oven, the samples from the shoot and root were ground in a Willey-type mill and passed through a sieve with a 2 mm mesh to conduct the Cu analysis in the tissues. This tissue was subjected to nitric-perchloric digestion (3.0 mL of HNO 65% p.a. and 1 mL of HClO 70% p.a.) (Embrapa, 2009). Then, the total Cu concentration was analyzed using an atomic absorption spectrophotometer (AAS, Perkin Elmer Analyst 200, USA).

2.6 Biochemical variables

Three plants were collected per replication for biochemical analyses, totaling 12 plants evaluated per treatment. When collected, they were washed with distilled water, placed in aluminum foil envelopes, and immediately deposited in a Styrofoam box with liquid Nitrogen (N). They were then placed in an ultra-freezer and frozen at 80°C until analysis was conducted.

Then, the samples were macerated with liquid N until they were in the form of a powder. They were weighed on a digital scale in quantities stipulated in the literature for each analysis, with 0.05 g of sample for determining pigments, 0.5 g for antioxidant enzymes, and 0.3 g for hydrogen peroxide. Then, the samples were homogenized, transformed into a homogeneous liquid with a specific buffer, centrifuged, and then the supernatant of each sample was analyzed.

The analysis of photosynthetic pigments, that is, the determination of the concentrations of chlorophyll *a*, chlorophyll *b*, and carotenoids in the shoot, were conducted according to the method of Hiscox, Israelstan (1979) and calculated from the equation of Lichtenthaler (1987). For the enzyme guaiacol peroxidase (POD), the method proposed by Zeraik *et al*. (2008) using guaiacol as a substrate was used. The analysis of the enzyme superoxide dismutase (SOD) was conducted using the spectrophotometric method described by Giannopolitis, Ries (1977). Hydrogen peroxide (H_2O_2) was determined according to Loreto, Velikova (2001), and the H_2O_2 concentration was expressed as μmol g^{-1} fresh weight.

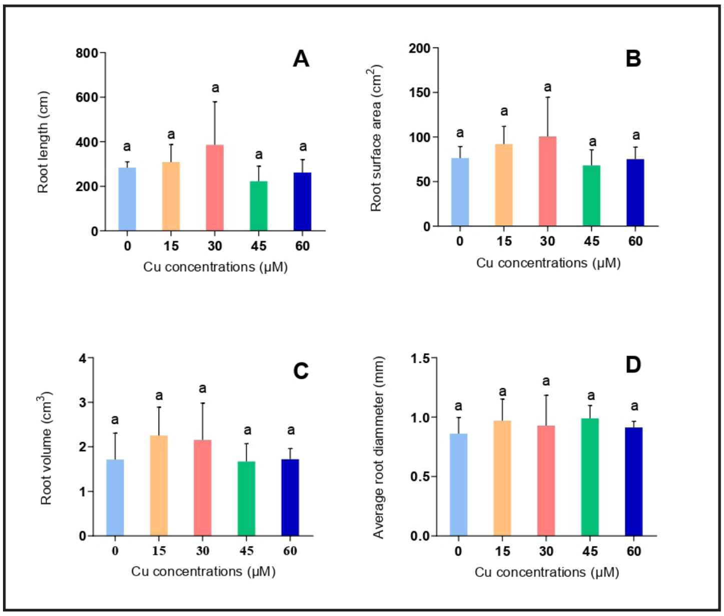
2.7 Statistical analysis

The data of the morphophysiological and biochemical variables were subjected to tests of homogeneity and normality and then submitted for the analysis of variance at a 5% probability level. The means were grouped by the Scott-Knott test at a 5% probability level. The statistical software SISVAR was used in the data analysis (Ferreira, 2019).

3 RESULTS AND DISCUSSIONS

Excess Cu, in general, negatively affects the morphological development of plants, such as root growth, as these are the first to come into contact with the metal, thus affecting the absorption of essential nutrients by the plant (Trentin *et al.,* 2022). However, in the present study, no significant differences were found for the variables of the root system of *C. americana* seedlings, such as total length, surface area, volume, and diameter of roots (Figure 1A, B, C, and D). On the other hand, an increase in root growth could be observed at intermediate Cu concentrations (Figure 2). This indicates that the seedlings of the species, in general, were not affected by the increase in Cu concentrations, and they may present tolerance to Cu.

Figure 1 – Average values of length (A), surface area (B), volume (C) and average root diameter (D) of *C. americana* plants grown in different concentrations of Cu in nutrient solution



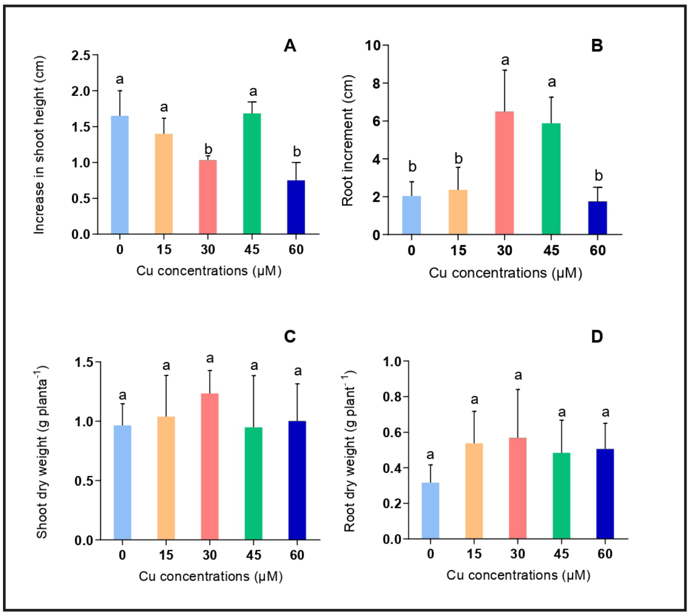
Source: Authors (2023)

In where: [1]Different letters between treatments represent statistical difference using the Scott-Knott test. Bars represent mean ± standard deviation.

For the variable increase in shoot height (Figure 2A), concentrations of 30 and 60 µM Cu demonstrated a significant difference, decreasing their averages concerning the control. The increase in main root length (IR) (Figure 2B) presented the highest averages at 30 and 45 µM Cu.

Despite the changes in SHI and IR promoted by Cu (Figures 2A and 2B), no significant change was observed in the biomass production of the plants (Figures 2C and 2D). This may have occurred due to the increase in the intracellular production of organic acids (citrate and phytochelatins), which act in the cytosol by chelating metal ions sequestered by the vacuoles, preventing greater effects on the plant (Aguilar *et al.*, 2023). According to Marques (2016), excess of this metal reduces root biomass, which was not seen in this study, indicating a possible tolerance of the species to excess Cu in the growth medium.

Figure 2 – Average values of Increase in shoot height (SHI) (A), root increment (IR) (B), shoot dry weight (SDW) (C) and root dry weight (RDW) (D) of *C. americana* plants grown in different concentrations of Cu in nutrient solution

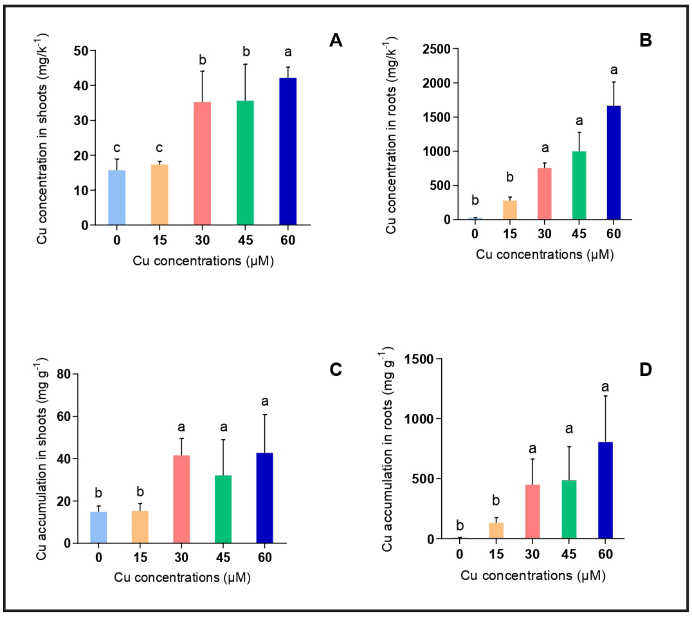


Source: Authors (2023)

In where: [1]Different letters between treatments represent statistical difference using the Scott-Knott test. Bars represent mean ± standard deviation.

The Cu content in the tissues of the shoot (Figure 3A) and root of *C. americana* (Figure 3B) increased with increasing Cu concentrations in the solution. Compared with the control, there is a greater Cu accumulation in the plants grown in nutrient solutions with 30, 45, and 60 µM Cu (Figures 3C and 3D). Therefore, Cu accumulation in tissues was more intense in the root system than in the shoot of *C. americana* plants. This can be explained by excess Cu being maintained in the apoplast attached to the cell wall or in the vacuole of root cells; consequently, there was a decrease in Cu translocation to the shoot (Aguilar *et al.,* 2023). The protection or lower concentration of heavy metals translocated to the leaves is a defense mechanism since this is the most metabolically active organ of the plant, responsible for photosynthesis (Aguilar *et al*., 2023). According to Covre *et al*. (2020), the higher concentration of metals in the root system is a necessary characteristic for phytoremediation species, suggesting their tolerance. Therefore, *C. americana* demonstrated a defense capacity aimed at protecting its shoot, similar to what was found by Covre *et al*. (2020) with the *Cedrella fissilis* and Kuinchtner *et al*. (2023) with *Handroanthus heptaphyllus*, where the species proved to be tolerant to Cu, with the technique of accumulating the metal in its root system, called phytostabilization (Kuinchtner *et al*., 2023).

Figure 3 – Average values of Cu concentration in shoot (A) and roots (B), and Cu accumulated in the shoot (C) and roots (D) in *C. americana* plants grown in different concentrations of Cu in nutrient solution



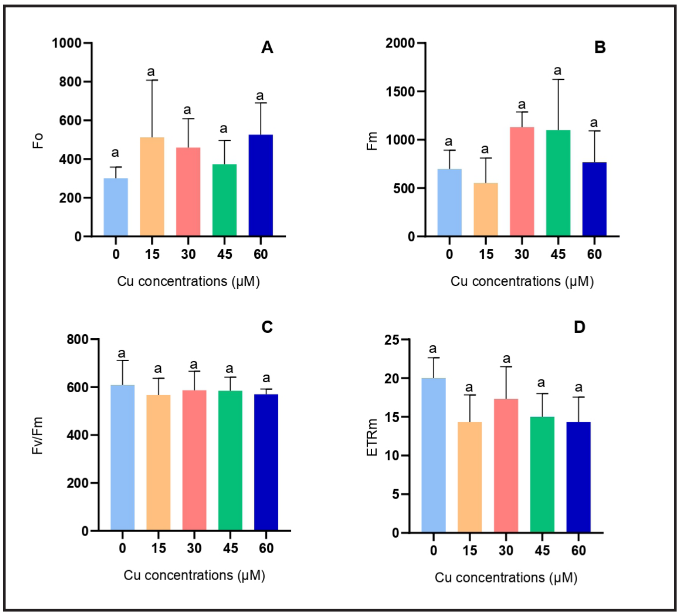
Source: Authors (2023)

In where: [1]Different letters between treatments represent statistical difference using the Scott-Knott test. Bars represent mean ± standard deviation.

High Cu concentrations are harmful to plants. They can present visible symptoms, such as chlorosis caused by damage to the thylakoid (Marques, 2016) membranes and reduced growth due to difficulty absorbing nutrients from the roots (Borghi *et al*., 2008). The photosynthetic rate, in general, is excessively affected and sensitive to stress caused by heavy metals and can be used as a reliable marker for stress (Kuinchtner *et al*., 2021). However, the symptoms above were not evident for *C. americana*.

For the initial fluorescence (Fo) (Figure 4A), maximum fluorescence (Fm) (Figure 4B), maximum quantum yield of photosystem II (Fv/Fm) (Figure 4C), and electron transport rate (ETR) (Figure 4D), it was observed that *C. americana* plants were not significantly affected by the presence of Cu in the nutrient solution compared to the control treatment.

Figure 4 - Average values of initial fluorescence (Fo) (A), maximum fluorescence (Fm) (B), maximum quantum yield of PSII (Fv/Fm) (C) and electron transport rate (ETRm) (D) and in *C. americana* plants grown in different concentrations of Cu in nutrient solution

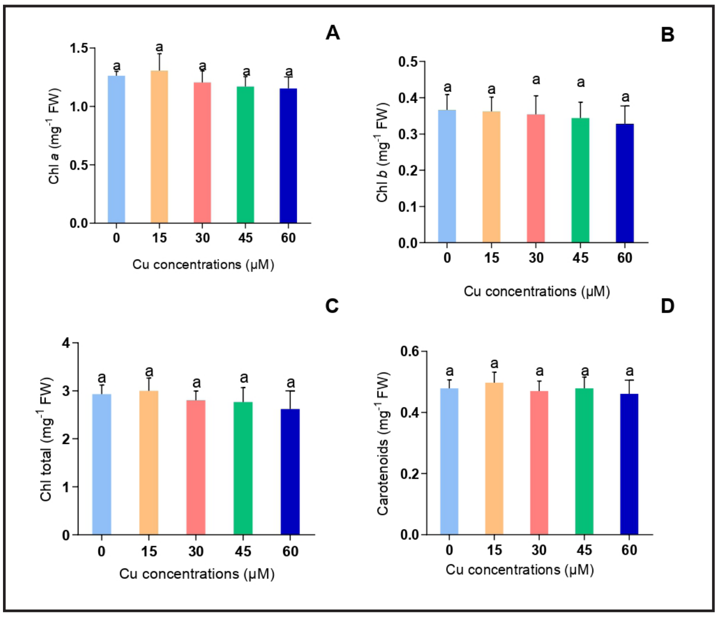


Source: Authors (2023)

In where: [1]Different letters between treatments represent statistical difference using the Scott-Knott test. Bars represent mean ± standard deviation.

The values found for chlorophyll *a* (Figure 5A), chlorophyll *b* (Figure 5B), carotenoids (Figure 5C), and total chlorophyll (Figure 5D) of *C. americana* plants were not influenced by increasing Cu concentrations. Excess Cu did not affect the content of photosynthetic pigments and the variables related to photosynthetic rates (chlorophyll a fluorescence). This explains why biomass production was not negatively affected by excess Cu, reinforcing the high tolerance of *C. americana* to Cu stress. According to Borghi *et al*. (2008), plants tolerant to heavy metals must show increased biomass production or not statistically differ from the control when exposed to heavy metals. In the study by Oliveira (2020), the *Mimosa scabrella* species did not have its photosynthetic pigment content affected by the increase in cadmium concentrations in the nutrient solution. The author concludes that cadmium did not negatively affect the photosystems and the synthesis of molecules, which resulted in desirable characteristics for phytoremediation. Thus, it can be understood that *C. americana* plants exposed to Cu maintained their pigment production even with additions of Cu, which corroborates the fact that Cu accumulation was greater in the root system and lower in the shoot so that pigment production would not be affected.

Figure 5 – Average values of Chlorophyll *a* (A), Chlorophyll *b* (b), Carotenoids (c) and total Chlorophyll (d) in *C. americana* grown in different concentrations of Cu in nutrient solution



Source: Authors (2023)

In where: [1]Different letters between treatments represent statistical difference using the Scott-Knott test. Bars represent mean ± standard deviation.

In general, an excess of this heavy metal causes an increase in the production of highly toxic oxygen-free radicals. These free radicals, reactive to oxygen, are known as superoxide radicals ($O2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{-\bullet}$)

(Aguilar *et al*., 2023), which damage lipids, nucleic acids, and proteins, among others (Marques, 2016). Therefore, the plant uses strategies to combat oxidative stress through enzymes such as superoxide dismutase (SOD) and guaiacol peroxidase (POD), which act by sequestering reactive oxygen species (ROS).

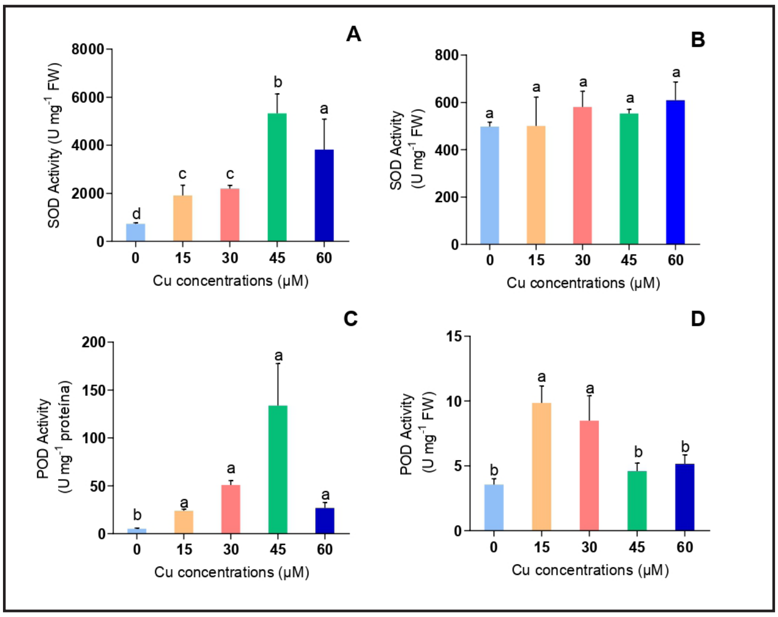
SOD is an enzyme that catalyzes the dismutation of superoxide radicals into O_2 and H_2O_2. At the same time, POD converts H_2O_2 into water and oxygen by dissociation of H_2O_2, which is important for tolerance to unfavorable conditions in plants (Aguilar *et al*., 2023). Furthermore, SOD helps in the formation of H_2O_2 (Ambrosini *et al*., 2016) as this is the least harmful ROS, compared to the others, and is combatted by the action of POD. Although H_2O_2 plays a key role in the growth and development of plants by regulating the opening of stomata, photosynthesis, and protection from abiotic stress, when the plant produces it in higher concentrations, it becomes harmful, increasing lipid peroxidation (Aguilar *et al*., 2023).

Given the above, the activity of the antioxidant enzyme superoxide dismutase (SOD) and the activity of the enzyme guaiacol peroxidase (POD) in the shoot (Figures 6A and 6C), increased with the addition of Cu, compared to the control. On the other hand, it was observed that SOD activity in the roots (Figure 6B) did not present a significant difference compared to the control, regardless of the concentrations of Cu applied in the nutrient solution. However, the POD for the roots (Figure 6D) increased only at concentrations of 15 and 30 μM Cu, compared to the control.

The shoot of *C. americana* seedlings exhibited increased antioxidant enzyme activity in the presence of Cu and increased H_2O_2 content (Figures 5 and 6). Although H_2O_2 levels in the shoots (Figure 7A) increased, there was no reduction in biomass production, indicating that activating antioxidant enzymes was sufficient to prevent damage to cellular constituents. Furthermore, no reduction in biomass production was observed in the presence of Cu in *C. americana* plants, which may indicate the non-occurrence of significant oxidative damage to cellular constituents.

The increase in H_2O_2 content at concentrations of 15 and 60 μM Cu for the roots (Figure 7B) of *C. americana* cannot be explained by the increase in SOD activity since it was not significantly affected by the concentrations of Cu. Furthermore, even with POD being activated, H_2O_2 levels were not reduced in the roots, but the biomass production of these tissues was not negatively affected by Cu. Therefore, the increase in H_2O_2 was not enough to cause oxidative damage.

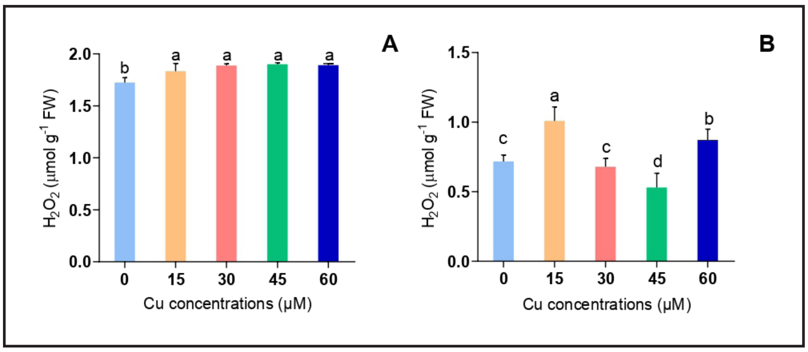
Figure 6 – Average values of superoxide dismutase (SOD) enzyme activity in shoots (A) and roots (B), and guaiacol peroxidase (POD) enzyme activity in shoots (C) and roots (D) in plants *C. americana* grown in different concentrations of Cu in nutrient solution



Source: Authors (2023)

In where: [1]Different letters between treatments represent statistical difference using the Scott-Knott test. Bars represent mean ± standard deviation.

Figure 7 – Average values of hydrogen peroxide (H_2O_2) content in shoots (A) and roots (B) of *C. americana* grown in different concentrations of Cu in nutrient solution



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In where: [1]Different letters between treatments represent statistical difference using the Scott-Knott test. Bars represent mean ± standard deviation.

4 CONCLUSIONS

From the increase in Cu in the nutrient solution, an increase in the accumulation of the metal in the plant tissues of the shoot and roots can be observed. Even with the highest concentrations of Cu accumulated in its organs, the species did not reduce biomass production.

As one of the strategies, the species maintained higher concentrations of Cu accumulated in its root system, allowing the shoot to continue photosynthesis without compromising the growth of the shoot and roots.

Therefore, the *Cordia americana* species is tolerant to Cu concentrations and can be recommended for phytoremediation of soils contaminated with this metal. Morphophysiological and biochemical variables can be used to select tolerant species to Cu toxicity. Furthermore, data from this study can serve as a basis for future research on the action of toxic elements on plants at a field scale, which can be an effective approach for new assessments of the phytoremediation potential of the species.

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Authorship Contribution

1 Luana da Rosa Lovato

Forestry Engineer student

https://orcid.org/0000-0003-1700-6401 • luanarslov@gmail.com

Contribution: Formal analysis; Investigation; Methodology; Project administration; Software; Supervision; Writing - original draft; Writing - review & editing

2 Marcos Vinícius Miranda Aguilar

Forest Engineer, PhD student in the Postgraduate Program in Forestry Engineering

https://orcid.org/0000-0003-0480-6119 • aguilarmarcos2009@hotmail.com

Contribution: Conceptualization; Data curation; Methodology; Writing – original draft; Writing – review & editing

3 Flaiane Catarine Rosa da Rosa

Forestry Engineer student

https://orcid.org/0000-0002-8904-6632 • flaianecatarine@hotmail.com

Contribution: Data curation; Formal analysis; Investigation; Methodology

4 Thomas Wink Peixoto

Biologist

https://orcid.org/0000-0003-2521-8746 • thomaswinkpeixoto@gmail.com

Contribution: Data curation; Formal analysis; Investigation; Methodology

5 Gerâne Silva Wertonge

Forest Engineer, PhD student in the Postgraduate Program in Forestry Engineering

https://orcid.org/0000-0001-7695-3789 • gerane-wertonge@hotmail.com

Contribution: Data curation; Formal analysis; Investigation; Methodology

6 Daniel Vinicios Valsoler

Biological Sciences student

https://orcid.org/0000-0002-5648-3035 • danielvalsoler15@gmail.com

Contribution: Data curation; Formal analysis; Investigation; Methodology

7 Tais Dorneles de Azevedo

Biological Sciences student

https://orcid.org/0000-0002-0325-9339 • thais.azevedo@acad.ufsm.br

Contribution: Data curation; Formal analysis; Investigation; Methodology

8 Lucas Caye da Silva

Forestry Engeneer student

https://orcid.org/0000-0003-4221-0447 • lucascaye06@gmail.com

Contribution: Data curation; Formal analysis; Investigation; Methodology

9 Luciane Almeri Tabaldi

Professor, PhD in Agronomy

https://orcid.org/0000-0002-3644-2543 • lutabaldi@yahoo.com.br

Contribution: Funding acquisition; Project administration; Resources; Supervision; Writing – original draft; Writing – review & editing

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